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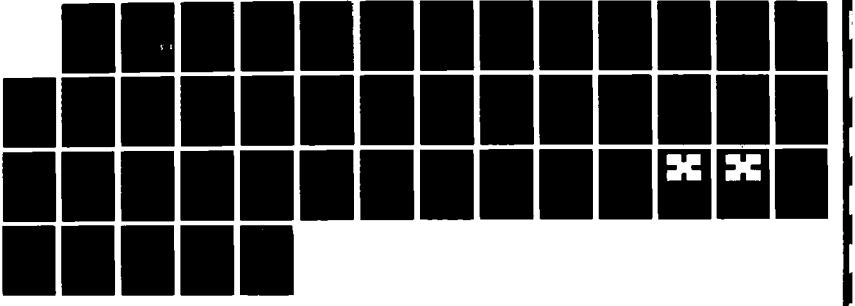

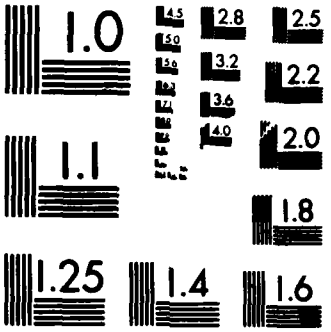

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Efficacy and Mode of Action of Immune Response Modifying Compounds Against Alphaviruses and Flaviviruses

Annual Report

Page S. Morahan, Ph.D.
Margo Brinton, Ph.D.
Angelo Pinto, Ph.D.

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The Medical College of Pennsylvania
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SUMMARY

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* expanded antiviral agents

FOREWORD

Citations of commerical organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products of services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

INTRODUCTION

This research is directed at two of the areas of interest for developmental research on immune modifying compounds against viruses of military importance.

1. Evaluate compounds for modulation of specific and nonspecific immune responses in normal B6C3F1 female mice. We are using drug treatment regimens that have been shown to be effective in the in vivo antiviral tests by us and others. We have focused on in vitro/ex vivo tests for intrinsic and extrinsic macrophage antiviral activity, macrophage activation, and natural killer cell activity.

2. Establish the efficacy of prophylactic and/or therapeutic treatment with immunomodulators alone and in combination with antiviral drugs against alphavirus, flavivirus, bunyavirus and herpesvirus infections in vivo. Both peripheral and intracerebral inoculation of viruses have been used, in order to assess drug efficacy against systemic infection and encephalitic disease. We are evaluating and characterizing the antiviral efficacy in normal mice, and we are evaluating a few regimens of particular interest in mice that have been selectively depleted of various nonspecific effector cell populations.

BACKGROUND

The need for better control of the "exotic" RNA viruses which are members of the Alphaviridae, Flaviviridae, Bunyaviridae, and Arenaviridae is well documented. Complete control will undoubtedly require a multifaceted approach, including better insect and rodent vector control, effective vaccines, and development of safe and effective prophylactic/therapeutic treatment. Development of antiviral treatment for RNA viruses has lagged behind drug development for DNA herpesviruses, where we now have the second generation of active nucleoside analogues (7,14). Considerable research in recent years, however, has documented that many of the exotic RNA viruses are inhibited by a variety of immunomodulators, including interferon, as well as several novel nucleosides (7,41). Importantly, combinations of two different nucleosides or immunomodulators have clearly been shown to produce synergistic effects. Most of these studies have been performed only in vitro (14,20). There is evidence, however, of synergy in vivo for ribavirin combined with the interferon inducer poly IC-LC against Rift Valley fever (RVF) infection in mice (17), and for ribavirin and specific antibody against Lassa fever virus in monkeys (15). Antiviral activity against RVF in mice can also be enhanced if nucleosides such as ribavirin or immunomodulators such as muramyl dipeptide (MDP) are delivered in liposomes (7,16).

Neither the predominant mechanisms of action of these various drug combinations, nor the optimum treatment regimens (dose, schedule, route) have yet been established. It has been well documented that immunomodulators can exert "ying-yang" effects on host resistance varying with the compound injection dose, route and schedule in relation to virus or tumor challenge (29,30). Nucleosides have toxic properties (8,9) and can also alter the lymphoreticular/hematopoietic system and thus alter the toxicity or the protective action of immunomodulators (11). Thus, systematic evaluation of antiviral efficacy and immunomodulatory activity of promising compounds and compound combinations is necessary before the best clinical treatment can be developed.

For these reasons, the current study has evaluated different regimens of immunomodulator treatment for antiviral efficacy against exotic RNA viruses and has attempted to correlate antiviral protection with antiviral effector mechanisms. Specifically, we have focused on drug-induced changes in macrophage (MØ) activation, as assessed by antitumor activity and ectoenzyme phenotype, and natural killer cell (NK) activity, mechanisms which have been well documented to be important in resistance to virus infections (3,4,24,25,33). Our results demonstrate that several immunomodulators modulate nonspecific resistance and are effective antiviral agents in vivo. Thus, these drugs may be useful in prophylactic or early therapeutic treatment of several serious viral infections. Whether the antiviral mechanism(s) of action is related to the effects on the natural immune system will be a major thrust of our future studies.

RATIONALE

A variety of potentially useful immunomodulators are being characterized for immunomodulatory activity in the B6C3F1 female mouse animal model that is widely accepted for immunotoxicological and immunopharmacological preclinical evaluations. A battery of standardized immune function assays is being performed. We are focusing especially on MØ and NK cell activation (28). The antiviral activity of MØ against four classes of exotic RNA viruses (alpha-, flavi-, bunya- and arena-viruses) is being studied to establish if MØ exert broad spectrum antiviral activity through common antiviral mechanisms. The study of novel immunomodulators in standardized assays, in comparison with "classic" immunomodulators, allows assessment of the most probable effective immunomodulatory mechanisms of the new compounds.

The second focus of the present research is the establishment of the antiviral efficacy of immunomodulators against a variety of exotic RNA virus infections. For comparison, antiviral efficacy against HSV-2 infection is used as the "gold standard", because the mechansims of natural host resistance and protective immunomodulatory treatment against HSV have been considerably characterized. A spectrum of RNA virus infections has been selected for study, in order to establish if broad spectrum protection can be produced against viruses that possess quite different replication and viral pathogenesis patterns. The infection models are usually initiated parenterally, because antiviral treatment should be most efficacious in this setting. The models selected include intraperitoneal infections with: Semliki forest L10 alphavirus, Banzi flavivirus, and Oriboca or Caraparu bunyaviruses. These viruses produce significant mortality in adult mice after i.p. infection, but can be worked with under Class II biohazard conditions. Three models of intracerebral infection were selected for evaluation in situations where it is important to establish if a given treatment can be effective when virus is already present in the CNS. The models include: Semliki forest alphavirus, Yellow Fever flavivirus, and La Crosse bunyavirus.

In summary, the rationale for this research is that: (i) better methods are needed for treatment of exotic RNA virus infections; (ii) immunomodulation provides a potent new modality of antiviral prophylaxis and therapy; (iii) but, immunomodulation can also produce adverse effects; (iv) and thus, there is a need for systematic evaluation of immunomodulators against diverse viral infections, together with more precise delineation of their mechanisms of action on nonspecific immune effector cells and immunoregulatory networks under carefully controlled standard systems.

EXPERIMENTAL METHODS

Mice. Virus free, barrier raised, 6 week old female B6C3F1 mice were purchased from Ace Animals Inc., shipped in filter crates and housed in autoclaved micro-isolator cages (MCP) or PLAS - LAB isolator chambers (Wistar). Usually, two mice from each shipment were bled on arrival, two more at one week, and periodically thereafter for testing to ensure no inapparent viral infections had occurred. Mouse sera were tested for sero conversion to MHV and Sendai viruses by the ELISA test (Biocon Labs, Rockville, MD).

Immunomodulators: Table 1 lists the immunomodulators used in this study. C. parvum (Burroughs Wellcome Co., Research Triangle Park, NC) was injected into mice i.p. at 35 mg/kg 7 days prior to cell harvest or infection of mice. MVE-2 (courtesy of D. Breslow, Hercules, Inc., Wilmington, DE) was dissolved in phosphate-buffered saline to a final inoculation concentration of 50mg/kg and was administered i.p. to mice 1 day prior to virus infection. TDM and MPL (Ribi Immunochem Research Inc., Hamilton, MT) were solubilized in 2% squalene at 56°C and injected i.p. MTP-PE (Ciba Geigy, Basle, Switzerland) was administered i.p. in liposomes (preparations kindly provided by Dr. David Gangemi). rIFN-G (courtesy of Genentech, San Francisco, CA), rIFN-A (courtesy of Hoffman LaRoche, Nutley, NJ) and rTNF-A (courtesy of Genentech) were injected i.p. in a vehicle of phosphate-buffered saline containing 0.2% bovine serum albumin (BSA). Ampligen (courtesy of Dr. Paul Tso, Johns Hopkins, Baltimore, MD) was dissolved in purified sodium chloride, heated at 67°C for 16 hours then at 37°C for 1 hour before being injected i.p. CL246,738 (courtesy of Lederle, Pearl River, NY) was prepared in distilled water and administered per os.

Viruses. Each virus pool was prepared somewhat differently, in order to maximize the titer of virus produced. The in vitro and in vivo titers of the various viruses are presented in Table 2. Herpes simplex viruses type 1 (HSV-1 Kos) and type 2 (HSV-2 MS) were prepared in either secondary rabbit kidney fibroblasts or Vero cells, by infecting cells with a low multiplicity of infection (m.o.i.), and harvesting the cultures when more than 75% of the cells showed cytopathic effect (CPE) (26). The pools consisted of cell-associated virus that was clarified of cellular debris by low speed centrifugation. Both HSV-1 and HSV-2 were titered on Vero cells with 2% methylcellulose overlay, and the titers were about 2×10^8 plaque forming units (PFU/ml) for HSV-1 and 8×10^6 PFU/ml for HSV-2.

Pools of alphaviruses, flaviviruses, and bunyaviruses were made in newborn CD-1 mice. In all cases two to four day old mice were inoculated with 0.02 ml of virus by the intracerebral route. When moribund, the mice were sarificed by decapitation and their brains or livers removed and frozen on dry ice. Subsequently, clarified 10% (wt/vol) tissue homogenates were made, aliquoted, and stored frozen at -70°C.

A baby hamster kidney (BHK) tissue culture pool of the alphavirus Sindbis, strain SAAR339, was used to infect mice at 1.2×10^7 PFU/mouse. The mouse brain pool that was obtained had a titer of 2.5×10^9 PFU/ml on BHK-21 cells, but did not kill mice. In an attempt to enhance the ability of this virus to kill adult mice, a second passage mouse brain pool was prepared. Mice were inoculated with 5×10^6 PFU/mouse of the first brain pool and sacrificed 20 hr after infection. This pool had a titer of 5×10^9 PFU/ml but did not kill adult mice.

A seed stock of alphavirus VEE, TC83 vaccine strain, Lot 4 run 2, USAMRIID, was obtained from Dr. Robert Shope, Yale Arbovirus Research Unit. Mice were inoculated with a 1:10 dilution as described above. Mice were sacrificed 69 hr after infection. A brain pool was prepared. Virus infection was assayed by plaque titration in BHK-21 cells (titer = 1.3×10^{10} PFU/ml). In order to meet containment guidelines for the experimental use of human pathogens, we have decided to discontinue experiments with VEE. In the future, L10 strain of Semliki Forest virus (SFV) (from Dr. Joel Dalrymple) will be used for in vivo and in vitro experiments as our alpha togavirus model.

The flavivirus, Yellow Fever virus, 17D vaccine strain, was obtained from Connaught Laboratories. Mice were inoculated with 2×10^2 PFU/mouse and were moribund by 7 days after infection. A brain pool was prepared and contained a titer of 1.6×10^7 PFU/ml as assayed on BHK21 cells.

A seed stock of the flavivirus, Banzi virus, strain SA M336 mouse passage 9, was obtained from Dr. Shope. Mice were inoculated with a 1:2 dilution of this stock. Mice were sacrificed 3 days after infection. The brain pool prepared contained an infectivity titer of 2.0×10^8 PFU/ml as assayed by plaque titration on BHK 15 cells. A second passage brain pool was prepared. Mice were inoculated with 4.8×10^4 PFU/mouse and sacrificed 48 hr after infection. This pool had a titer of 1.5×10^8 PFU/ml.

A seed stock of a second mouse passage of the bunyavirus, Caraparu, strain Be AN 3999, was obtained from Dr. Shope. Mice were inoculated intracerebrally with a 1:100 dilution of this stock and sacrificed 48 hr after infection. The livers were removed and used to prepare a clarified 10% (wt/vol) liver homogenate. No plaques were obtained with this virus on BHK-21, BHK-15 or MK_2 cell monolayers. The pool had an LD_{50} of $10^{2.33}$ in adult CD-1 mice inoculated by the i.p. route.

A seed stock of the bunyavirus, Oriboca, strain BeAn17, mouse passage 12, was obtained from Dr. Shope. Mice were inoculated with a 1:100 dilution of this stock and sacrificed 48 hr after infection. A 10% liver pool was prepared and had an infectivity titer of 4×10^6 PFU/ml as assayed on BHK-21 cells.

Target Cell Lines. Two different cell lines were used in this study. These were: Lewis lung (LL), a carcinoma derived from spontaneous tumors in C57BL/6 mice (35); and YAC-1, a T cell lymphoma derived from the A/SN mouse (13). LL was maintained as monolayers in $25cm^2$ plastic flasks with Eagles's minimum essential medium (EMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine calf serum (FBCS) (Flow Laboratories, McLean, VA), 2% MEM vitamins (Flow), 1% non-essential amino acids (Flow), 1% essential amino acids (Flow), 1mM sodium pyruvate (Flow), and 25mM HEPES buffer (Sigma). For convenience, this culture medium hereafter will be called complete EMEM. YAC-1 cells were maintained in stationary suspension cultures in $25cm^2$ plastic flasks with RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FBCS (Flow), 1% non-essential amino acids (Flow), 1mM sodium pyruvate (Flow), and 25 mM HEPES buffer (Sigma). For convenience, this culture medium hereafter will be called complete RPMI.

Peritoneal MØ Preparation. MØ were harvested by flushing the peritoneal cavity with 5ml of cold Dulbecco's phosphate-buffered saline (DPBS) (Gibco)

containing 2 units/ml preservative-free heparin (Invenex Laboratories, Melrose Park, IL), and 50ug/ml gentamicin (Gibco). The peritoneal cells were counted in a Model ZM Coulter Counter (Hialeah, FL), centrifuged at 250 x g for 10 minutes at 5°C and resuspended to a concentration of 2.5×10^6 cells/ml in complete EMEM containing 50ug/ml gentamicin. Slides for cell differentials were prepared in a Cytospin (Shandon-Southern, Sewickley, PA) and were stained with a modified Wright's stain (Geometric Data, Wayne, PA).

Preparation of Spleen Cells. Spleens were removed, trimmed of fat and placed in 10ml manual glass tissue grinders containing 5ml cold RPMI 1640. Spleen cells were dispersed into the RPMI 1640 by gentle grinding, and the resulting suspension was transferred to a 17 x 100 mm round bottom plastic centrifuge tube. After an initial wash, contaminating red blood cells were removed by resuspending the cell pellet in distilled water for 20 seconds followed by an equal volume of 2 x DPBS. Cells were then washed again at 250 x g for 10 minutes before being resuspended to 2×10^7 cells/ml in complete RPMI.

MØ Ectoenzyme Assays. Peritoneal cells were allowed to adhere in 35mm-diameter well plates at a concentration of 2.5×10^6 cells/ml/well for 2 hours at 37°C in a 5% CO_2-air mixture. Afterwards, nonadherent cells were removed by three washes with cold DPBS. The adherent cells were lysed with 0.05% Triton-X-100 in distilled water (200ul/well). The lysates were frozen at -20°C until assayed, using 100ul for alkaline phosphodiesterase I (APD), 20 ul for 5' nucleotidase (5'N), and 60 ul for protein determination. Protein concentration was determined using the Bio-Rad procedure (Bio-Rad Laboratories, Rockville Center, NY).

5'N specific activity (S.A.) was determined using 0.15 mM ^{3}H-adenosine monophosphate (AMP) as the substrate and p-nitrophenyl phosphate as the competitive inhibitor of phosphatase activity (23). The S.A. was expressed as n moles AMP hydrolyzed per minute per mg protein at 37°C. APD S.A. was assessed as previously described (23) using 1.5 nM p-nitrophenyl thymidine -5'-monophosphate as the substrate. The S.A. was determined using the extinction coefficient 12000 for p-nitrophenol and was expressed as n moles of p-nitrophenol produced per milligram protein per minute at 37°C.

MØ Antitumor Assay. An adaptation of the densitometric micromethod developed by Leu and Herriott (19) was employed. Peritoneal cell suspensions were diluted to 1.2×10^6 MØ/ml and added to test wells of flat bottom microtiter plates in one of the following amounts: 200ul, 100ul, and 50ul; these corresponded to effector to target cell ratios of 20:1, 10:1 and 5:1 respectively. All wells were brought to a final volume of 200ul with complete EMEM. After 2 hours of incubation at 37°C, nonadherent cells were washed off with cold DPBS and a 200ul aliquot of LL target cells (1.2×10^4 cells/well) was added to the appropriate wells. To facilitate an even cell distribution, test plates were centrifuged at 55 x g for 5 minutes. Plates were then incubated for 48 hours at 37°C in a 5% CO_2-air mixture. Following incubation the supernatant fluids were decanted and the cells were washed gently with warm DPBS, fixed for 30 minutes in 33% absolute ethanol containing 1.3% formaldehyde in saline and stained with 0.5% crystal violet for one hour (32). The plates were washed, air dried and the stained monolayers were solubilized with 50% ethanol in distilled water for one hour. Tumor cell cytotoxic/cytostatic activity was determined by measuring the amount of light (570nm) transmitted through the wells using a MR 580 Microelisa Auto Reader (Dynatech, Alexandria, VA). Tumor cell destruction

was calculated according to the following formula:

$$1 - \frac{(OD_{570} \text{ of M}\emptyset + \text{target cells}) - (OD_{570} \text{ of M}\emptyset \text{ alone})}{(OD_{570} \text{ of target cells alone})}$$

At least 3 wells/group were run and the average OD_{570} used.

NK Cell Assay. NK activity was assessed using a standard ^{51}Cr release assay (10). Tests were conducted in triplicate determinations in round bottom microtiter plates with varying amounts of effectors added to test wells in 100ul complete RPMI. Target YAC-1 cells were radiolabeled with 100 uCi $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA). Approximately 1×10^7 cells were labeled for 2 hours at 37°C with gentle shaking every 15 minutes. Labeling was terminated by the addition of cold RPMI 1640 and the cells were then washed twice before being resuspended in complete RPMI. Labeled targets (1×10^4) were then added to all wells to give a final volume of 200ul/well. To facilitate cell contact, test plates were centrifuged at 55 x g for 5 minutes. Following incubation at 37°C for 4 hours, plates were centrifuged at 500 x g for 10 minutes at 5°C. A 100ul aliquot of supernatant was harvested from each well and placed in a plastic tube for counting in a gamma counter. The percent specific ^{51}Cr release was calculated from the following formula:

$$\% \text{ Specific } {}^{51}Cr \text{ release} = \frac{\text{Test release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \times 100$$

Test release = mean counts per minute (CPM) in supernate of wells containing ^{51}Cr labeled target cells plus effector cells.
Spontaneous release = mean CPM in supernate of wells containing ^{51}Cr labeled target cells plus complete RPMI.
Total release = mean CPM in supernate of wells containing ^{51}Cr labeled target cells plus an equal volume of the detergent Triton-X-100 diluted 1:5 in distilled water.

Intrinsic MØ Virus Interactions. Resident (Res) peritoneal MØ were obtained by our usual lavage procedures, washed and allowed to adhere for 2 hr. The adherent Res MØ were cultured for 24 hours before infection. For a few experiments, the Res MØ were cultured for 4 to 7 days and then infected. For infection with HSV, the number of MØ present was estimated by counting nuclei obtained by pronase cetrimide (34), and the amount of virus was adjusted to provide the appropriate m.o.i., which was usually about 3 to ensure infection of all the MØ. Virus was allowed to adsorb for 1 hr, nonadsorbed virus was removed by washing, and the MØ were cultured at 37°C. For some experiments, the amount of non-adsorbed virus was estimated by titrating the amount of virus in the supernatant fluid, and the amount of adsorbed but not yet eclipsed virus was estimated by titrating the amount of cell-associated infectious virus present at 1 hr. The MØ cultures were observed for cytopathic effect (CPE), and total (cell plus supernatant fluid) or supernatant virus samples were obtained and frozen until assayed by plaque titration. HSV samples were sonicated prior to titrating. Infectious virus yields were expressed as PFU per culture, and as PFU per cell in order to correct for differences in cell numbers in the

cultures. Various controls were usually included in the HSV MØ-virus experiments. A fully permissive cell line (such as Vero cells for HSV-1) was infected simultaneously with infection of Res MØ in order to ensure the viability of the virus preparation. A thermal inactivation control, consisting of the same concentration of virus as used in the MØ infection, was incubated to establish the rate of viral inactivation if MØ were not present.

For the infection of MØ with the RNA viruses, resident peritoneal cells were allowed to adhere for 3 hr to T25 flasks, to provide about 1.6×10^6 MØ/flask, and cultured for 24 hr. Viruses (about 0.003 - 3 moi) were allowed to adsorb to MØ for 1 hr, the excess inoculum was removed, and 5 ml media were added. At various intervals, 0.3 ml of the supernatant fluid was removed, frozen at -70°C, and titrated for PFU. The media was replaced with fresh media.

HSV-1 Viral Antigen Immunofluorescence Studies. Specific monoclonal antibodies (mab) to HSV-1 viral proteins were used on infected cell preparations to determine whether any expression of the viral proteins occurred. Vero cells and Res MØ populations were grown and harvested at various time points following infection with HSV-1. The coverslips were then fixed in acetone 15-20 min and air-dried. Immunofluorescence was assayed either directly, using fluoresceinated mab to VP5 (Kallestad Laboratories, Inc., Austin, TX) or indirectly, using mab to ICP4 (courtesy of L. Pereira) followed by fluoresceinated rabbit anti-mouse IgG (Miles Laboratories, Elkhart, IN). Results are expressed as the percentage of positive cells from a minimum of 300 cells counted.

Antiviral Protection Studies. B6C3F1 mice were usually randomized into experimental groups by using a computer generated random numbers series. Generally 15 mice were used in the placebo control group, and 10 mice in each experimental group. A small LD_{50} dose response was performed simultaneously with each experiment, in order to ensure that the appropriate number of LD_{50} doses was used for infection. Generally, a simultaneous *in vitro* titration of PFU/ml of the pool was also performed. Mice were infected i.p. for most experiments, and i.c. for a few. Mice were treated with the immunomodulators at the doses, routes and schedules indicated on each table of results. Infected mice were monitored daily for signs of clinical illness (ruffled fur, hunched back, paralysis, CNS symptoms) and mortality. Obviously moribund mice were sacrificed, and their day of death designated as the next day. Mice were observed usually for 14 days (Sindbis, Yellow Fever viruses) or for 21 days (HSV-2, VEE, Banzi, Oriboca, Caraparu). The percent mortality and mean survival days (MST) of each group were calculated.

In order to minimize data presentation, only the lowest doses that exhibited significant antiviral activity are reported. Where several experiments had placebo control groups that did not differ significantly in mortality or MST, the data for the control groups and appropriate experimental groups were combined, the mortality and MST were calculated, statistically evaluated, and presented.

Statistical Analysis. Statistical significance for the immunomodulator data was determined by the analysis of variance with Newman-Keul's multiple-range test with the level of significance set at p 0.5 (6). The mortality data were analyzed on an Apple IIe microcomputer using the Chi Square test included in the Applestat statistical package. This test is not overly conservative as is the

Fisher's exact test or Chi Square with Yate's correction factor, and thus can point out immunomodulator regimens with moderate activity (6). The MST was calculated on an Apple IIe microcomputer and compared with the appropriate placebo controls by the Mann Whitney U test (36).

RESULTS

Immunomodulator Profiles

Throughout the first year of this study, much of our work has focused on evaluating compounds for modulation of nonspecific immunity (MØ activation, NK cell activity). In our studies to characterize the immunomodulatory activity of these agents, we have used two positive controls: i.p. treatment of mice with 35 mg/kg C. parvum or 50 mg/kg MVE-2, two immunomodulators known to enhance nonspecific immunity (27). Use of these positive controls allows us to determine whether the mice are responding properly and also gives us a direct comparison of experimental immunomodulators with known drugs. When necessary we have used as negative controls both naive (untreated) mice and mice treated with the various drug vehicles, in order to control for possible immunomodulatory effects of the vehicles.

CL246,738. CL246,738 is an orally active compound known to augment nonspecific immunity (38,39). In a series of experiments we established the dose response and kinetics of CL246,738 induced changes in MØ activation and NK cytotoxicity. Results from the dose response study (Figure 1) indicate that in vivo treatment with CL246,738 at one day prior to cell harvest significantly enhanced splenic NK activity at doses ranging from 25 to 800 mg/kg, with peak lysis (about 63% lysis as compared to 25% in control mice) occurring at the 50 to 200 mg/kg doses. Similarly, peritoneal MØ antitumor activity against LL targets was significantly increased at all doses as compared to the naive control group. However, this enhanced activity was only moderate when compared to our positive control treatment, C. parvum (35 mg/kg, 7 days before sacrifice), which produced complete tumor cell destruction (data not shown). Data from the 5'N and APD ectoenzyme phenotypes (Table 3), indices which correlate with tumoricidal MØ functions (1,23), support the antitumor data in that CL246,738 induced only moderate declines in 5'N and APD as compared to the pronounced reduction observed in peritoneal MØ obtained from C. parvum treated mice.

A kinetic experiment with CL246,738 was performed at a 200 mg/kg dose on days -1, -4, -7 and -10 before cell harvest (Table 4). Again, splenic NK activity was highly augmented on the day after treatment (89.6% lysis as compared to 39.0% lysis in the control). Although the effect did diminish over time, NK activity was still significantly increased (61% lysis) 10 days after a single treatment. MØ antitumor data again indicated CL246,738 induces significant, but not potent, peritoneal MØ activation. (28,31 and 28% lysis/inhibition on days 4, 7 and 10 respectively after drug administration, as compared to 18% in naive mice and 67% in the C. parvum positive control group). A similar experiment with 800 and 400 mg/kg doses on 3 and 4 days prior to evaluating peritoneal MØ activation support our finding that CL246,738 is not a strong MØ activator, with low levels of antitumor activity being observed (Janauary 15, 1987 report) along with small decreases in 5'N and APD ectoenzyme activities (Table 3).

Ampligen. Table 5 illustrates the results of an initial dose response with the polyribonucleotide ampligen. As shown, ampligen was found to be a strong NK stimulant; significant levels of splenic NK lysis were seen even at a 1 mg/kg dose. Ampligen also induced moderate, but significant, levels of peritoneal MØ antitumor activity at an 8 mg/kg dose, an effect which gradually declined to a nonsignificant level at the 1 mg/kg dose. Ectoenzyme activities were significantly lower for both 5'N and APD in all treated groups; however, these reductions were not as pronounced as those in the C. parvum positive control mice.

Human Recombinant Gamma Interferon (rIFN-G). rIFN-G is a potent inducer of splenic NK activity against YAC-1 targets at doses as low as 30 U (Table 6). Likewise, rIFN-G is a good MØ activator as indicated by high levels of antitumor activity (comparable to C. parvum treated mice) with corresponding decreases in both 5'N and APD ectoenzyme activities. This latter finding is particularly significant since it extends our previous findings that alterations in ectoenzyme phenotype correlate with MØ tumoricidal activity (1,23). To the best of our knowledge, this observation of induced alterations in MØ ectoenzyme phenotype has not been reported before for gamma interferon.

Murine Recombinant Alpha A/D Interferon (rIFN-A). A dose response experiment was performed with rIFN-A by inoculating B6C3F1 mice i.p. with different levels of rIFN-A 24 hours prior to assessing splenic NK activity and peritoneal MØ antitumor activity and ectoenzyme phenotype. The results (Figure 2) indicate rIFN-A induces high levels of NK activity in a dose dependent fashion with plateau levels of 68% lysis reached at the 25,000 - 60,000 units/mouse dose as compared to 20% NK lysis in control mice. rIFN-A, however, produced only low levels of MØ antitumor activity, which was significant only at the 25,000 and 60,000 units/mouse dose. Peritoneal MØ 5'N activity in rIFN-A treated mice was not significantly altered, whereas APD activity was moderately, but significantly decreased in all but the 60,000 units/mouse group.

Detoxified Lipid A (MPL), Trehalose Dimycolate (TDM) and the Combination. We have also assessed MØ activation by treatment with MPL, TDM and the combination at 4 mg/kg and 8 mg/kg doses. MØ from untreated mice or mice treated with the squalene vehicle showed low levels of antitumor activity against LL targets (Table 7). Conversely, peritoneal MØ from the positive control group C. parvum had high levels of antitumor activity. At the 4 mg/kg dose all three experimental groups showed moderate-low levels of antitumor activity. 5'N specific activity was only markedly reduced in the combination group, whereas APD specific activity was reduced in all three groups. At the 8 mg/kg dose, TDM induced no MØ activation, whereas MPL caused a drop in ectoenzyme activities with no corresponding MØ antitumor activity. In contrast, combination treatment caused both increased MØ antitumor activity and decreased 5'N and APD ectoenzyme activities. Whether these differences in the 4 mg/kg and 8 mg/kg groups are dose dependent or are due to differences in response will be addressed in future studies.

Muramyl Tripeptide-Phosphatidylethanolamine (MTP-PE). We have performed two separate experiments, in collaboration with Dr. David Gangemi, on MØ activation by MTP-PE in liposomes. The results (Table 8) show that peritoneal MØ from control (naive) mice or mice treated with the pyrogen free NaCl vehicle had ectoenzyme phenotypes typical of resident peritoneal MØ. Treatment of mice with the positive control C. parvum caused strong decreases in both 5'N and APD

specific activity. Treatment with free MTP-PE caused moderate reductions in 5'N and APD. Injection of sham liposomes alone caused a marked reduction in 5'N but no change in APD. When sham liposomes and free MTP-PE were combined, 5'N was again reduced with no change in APD. Finally, mice treated with liposomes containing MTP-PE showed reduced APD in experiment 1 but not in experiment 2. In fact, in experiment 2 all groups that received liposomes showed marked enhancement of APD ectoenzyme activity. Whether these disparate results are due to technical variability or to variability in response to MTP-PE requires further investigation.

Intrinsic Interaction of MØ with Viruses. The intrinsic interaction of HSV-1 and HSV-2 with peritoneal MØ from B6C3F1 mice was compared with MØ from CD-1 mice. The interaction with Res MØ was compared with that of classic inflammatory MØ elicited with thioglycollate (TG MØ), and with C. parvum (CP) activated MØ. As we have shown in CD-1 mice (18,24,31), HSV-1 and HSV-2 did not replicate in Res MØ and produced little to no CPE (Fig. 3). The viruses also did not replicate in CP MØ, but did produce almost complete destruction of the MØ monolayer by 24-48 hr after infection. Some infectious virus was produced in the TG MØ, which also exhibited complete CPE. These three patterns of intrinsic interaction with HSV may be related to the different stages of activation or differentiation of these MØ. The permissiveness of human monocytes for HSV has previously been shown to vary with differentiation in vitro (12), and we have shown that a similar situation may exist with the U937 human MØ-like cell line (37). It will be important to determine whether immunomodulator activation of MØ leads to a general decrease in resistance to viral infection, as shown by increased viral growth or CPE. It is also evident that even the semi-permissive TG MØ are much less permissive than Vero cells, indicating the marked antiviral functions of MØ.

Initial experiments were performed with Res MØ infected with a variety of viruses. The present results are summarized in Table 9. These data emphasize the fact that Res MØ are generally quite resistant to replication with a variety of viruses. Usually declining titers of virus were measured with time after infection, and no viral CPE was observed. There were two instances where MØ that exhibited apparently complete resistance for infectious virus replication nevertheless showed CPE; these were CP MØ infected with HSV-1 or HSV-2 (Fig. 3) and Res MØ infected with YFV (Table 9). Whether this CPE is virally induced, or is a nonspecific result of treatment with the virus preparations, is still uncertain. We have shown that live HSV is required for production of CPE in TG MØ (unpublished observations). However, we have also observed that sometimes CPE can occur when undiluted or close to undiluted pools of virus are used for infection of MØ; it is possible that there are some nonspecific cytotoxins in these preparations (unpublished observations).

The fact that no production of infectious virus or CPE can be observed does not necessarily mean that no viral specific macromolecular synthesis occurs in Res MØ. We have shown that the HSV-1 immediate-early protein (ICP4) and the delayed-intermediate protein (VP5) are produced in a considerable number of Res, TG and CP MØ within 6 hr after HSV-1 infection (Table 10) (Figures 4 and 5). However, there is no evidence of viral DNA synthesis as evidenced by measuring production of viral DNA (18) or production of the late protein glycoprotein C which requires DNA synthesis (unpublished observations). Whether there is a common mechanism of intrinsic antiviral resistance in Res MØ to the various DNA and RNA viruses remains to be determined.

Lack of Antiviral Effectiveness of Immunomodulators Against Intracerebral Alphavirus and Flavivirus Infection. Mice that were infected i.c. with Sindbis or Yellow Fever viruses showed a relatively delayed pathogenesis, with MST of approximately 8 to 10 days (Table 11). Prophylactic treatment with a variety of generally very effective immunomodulators, including C. parvum, MVE-2 and CL246,738 (27), was completely ineffective in reducing mortality or delaying death after i.c. infection. Repeated therapeutic treatment with gamma, beta or alpha interferon was also ineffective. There are several possible reasons for these results. The protective effects of the immunomodulators that were tested may have to be expressed before a virus is transmitted to the central nervous system. Alternatively, the drug or the antiviral mechanisms or effector cells it induces may not efficiently cross the blood brain barrier. In this regard, it would be of interest to evaluate the antiviral effectiveness of muramyl dipeptide which activates MØ, and which is reported to cross the blood brain barrier and induce slow wave sleep (2).

Evaluation of Immunomodulators Against HSV-2 MS Infection. Several experiments performed with HSV-2 infections revealed a spectrum of immunomodulatory antiviral activity in B6C3F1 mice that was similar to that which we have previously observed in CD-1 mice (27). Essentially complete protection against mortality was provided by single prophylactic treatment on the day prior to infection with our positive control MVE-2 and with CL246,738 down to 25 mg/kg, while partial protection was provided by ampligen (Table 12).

Essentially complete protection against HSV-2 infection was also provided by single prophylactic administration 7 days prior to infection with another positive control immunomodulator, C. parvum. Similar prophylactic treatment with a variety of single or combined microbially derived cell wall structures provided partial protection. These included trehalose dimycolate and monophosphoryl lipid A, which have been shown to have activity against influenza virus infection (21). Two microbial products, PA-PE and S-209, however, were ineffective.

Treatment of mice with alpha interferon provided complete antiviral protection against HSV-2. Combined prophylactic/therapeutic treatment twice daily at 66,000 IU for as little as day -1 to 2 days after infection was sufficient. Other experimental groups revealed no difference between single and twice daily treatment. The minimum effective dose and schedule remains to be determined; investigation in CD-1 mice revealed that repeated therapeutic treatment with less than 300 IU was effective (27). Additional comparative experiments with alpha, beta and gamma interferons will be performed.

Evaluation of Immunomodulators Against Systemic Infection with Banzi Flavivirus. The antiviral immunomodulator profile observed against Banzi was generally similar to that obtained against HSV-2 infection (Table 13). Single prophylactic administration with MVE-2, C. parvum or CL246,738 down to at least 12.5 mg/kg, produced essentially complete protection from mortality. Treatment with CL246,738 (200 mg/kg, p.os) could be delayed until 24 hr after infection, and still produce a significant increase in MST, but this partial effect was gone if treatment was delayed until day 3 after infection. Interestingly, p.os treatment was more effective than was i.p. treatment at 1 day after infection.

Treatment with ampligen appeared to be more effective against Banzi than against HSV-2 or VEE viruses (Tables 12 & 14). Single treatment with 4 mg/kg 1

hr after infection produced almost complete protection, as did repeated treatment for 6 days. Whether 4 mg/kg is the minimum effective single dose remains to be determined. Delaying treatment with 4 mg/kg until 1 day after infection completely abolished this effect, suggesting that the antiviral effect was necessary very early in infection.

As was observed with VEE and with HSV-2, therapeutic treatment with alpha interferon appeared to be more effective against Banzi than was treatment with gamma interferon. Repeated therapeutic treatment with gamma interferon (50,000 IU for 6 days after infection) was completely ineffective. In contrast, single treatment with 24,400 IU of alpha interferon 1 hr after Banzi infection provided significant protection, while repeated treatment for 6 days provided complete protection against mortality. Repeated treatment with alpha tumor necrosis factor did not provide any protection against Banzi virus.

Evaluation of Immunomodulators Against Systemic Infection with VEE Alphaviruses. Similar to observations with HSV-2 and Banzi, marked effectiveness of single prophylactic treatment with MVE-2 or with CL246,738 down to at least 100 mg/kg was observed (Table 14). Unlike the modest antiviral effect observed against HSV-2, single prophylactic treatment with ampligen was not effective against VEE.
However, repeated combined prophylactic/therapeutic treatment from day -1 to 2 days after infection was very effective, and repeated therapeutic treatment from day 0 to 6 days after infection was moderately effective against VEE.

Treatment with alpha or gamma interferons again revealed that alpha interferon was considerably more effective than was gamma interferon. Single therapeutic treatment with 24,400 IU of alpha interferon was ineffective, but repeated therapeutic administration of this dose on days 0 to 6 after VEE infection provided marked protection against mortality and increased the MST. Similar repeated therapeutic treatment with 18,000 IU of gamma interferon was completely ineffective against VEE, while 50,000 IU produced only a significant increase in MST. Combined prophylactic/therapeutic treatment from day -1 to day 6 after infection with gamma interferon, however, was quite effective. These data indicate that gamma interferon is only effective if administered before viral infection, and suggest that either the mechanism of antiviral action or drug pharmacokinetics differ between the two interferons.

Evaluation of Immunomodulators Against the Caraparu Bunyavirus. One experiment has been performed with Caraparu infection, using single prophylactic administration of immunomodulators that were shown to be quite effective in producing resistance against i.p. infection with HSV-2, Banzi flavivirus and VEE alphavirus (Tables 12-14). In contrast to the effectiveness against the other viruses, only moderate protection against Caraparu was produced with MVE-2 and CL246,738 (Table 15). MVE-2 provided the greatest protection, with a reduction in mortality from 100% to 40% as well as a significant increase in MST from 4.2 to 11.0 days. The modest effect with 100 mg/kg CL246,738 in significantly increasing the MST to 7.8 days was lost at 50 mg/kg, in contrast to the marked protection that was observed with the other three viruses with doses as low as 12.5 mg/kg. Whether the decreased protective ability of the immunomodulators is related to differences in viral pathogenesis in regard to target organs or rapidity of disease, to differences in sensitivity to interferon or other antiviral mediators, or to other parameters are issues that will be investigated.

DISCUSSION

A variety of immunomodulators were shown to be effective against herpes simplex virus and the alphavirus, flavivirus and bunyavirus infections that were tested. The same general degrees of effectiveness were found when comparing the various immunomodulators, although the viruses did differ in regard to general sensitivity. Banzi virus infection appears to be most sensitive to immunomodulatory antiviral effects, with HSV-2 and VEE being slightly less sensitive, and Caraparu infection being least sensitive. The most effective immunomodulators were MVE-2, C. parvum, CL246,738 and alpha interferon. Agents which produced marked antiviral activity included ampligen and gamma interferon. Moderate, but statistically significant, protection against HSV-2 was produced with various microbially produced cell wall materials, including trehalose dimycolate and muramyl dipeptide. These levels of antiviral effectiveness are similar to those that have been observed in previous studies in CD-1 mice against HSV-2 and encephalomyocarditis viruses (27). Thus, a variety of chemically diverse agents can, after prophylactic administration, produce broad spectrum antiviral protection.

As has been generally observed with immunomodulators, the greatest effect is observed with prophylactic treatment (27). It is encouraging to note that early therapeutic treatment with such drugs as CL246,738, was also partially effective, and that repeated therapeutic treatment as with the interferons was effective. All of these data support the general concept that immunomodulators are most effective during the early part of viral pathogenesis, before the specific immune response is generated, the virus has become sequestered in a privileged site such as the CNS, or too much infectious virus has been produced for natural resistance effector mechanisms to have an impact.

Whether there is a common mechanism of antiviral activity among the active immunomodulators remains to be established. Immunomodulator induction of alpha/beta interferon probably does not explain all the observed activity. The effective agents include alpha interferon itself, strong inducers of alpha/beta interferon (CL246,738 ampligen), but also agents that induce low to non-detectable interferon levels (MVE-2) (5,22). Likewise, potent MØ activation, at least as defined by antitumor activity and ectoenzyme profile criteria, does not appear to have a common mechanism. We have found that a single dose of the orally active compound CL246,738 induces moderate to low levels of peritoneal MØ activation, but is a potent splenic NK stimulant with elevated activity observed to at least a 25 mg/kg dose and for at least 10 days after treatment. The polyribonucleotide, ampligen, and alpha interferon were similar to CL246,738. Both ampligen and alpha interferon induced moderate to low levels of MØ activation, but were strong inducers of splenic NK activity even at low doses. In contrast to CL246,738, ampligen and rIFN-A, rIFN-G was an excellent MØ activator, as evidenced by MØ antitumor activity and ectoenzyme phenotypes comparable to the C. parvum positive control. rIFN-G was also an excellent inducer of NK cell activity, as has been reported previously (40). Despite these strong immunomodulatory effects on MØ and NK cells, however, rIFN-G was not as effective in producing broad spectrum antiviral protection as were CL246,738 and rIFN-A immunomodulators.

The immunomodulatory effects of the immunomodulators that had modest antiviral activity were inconsistent. Studies with TDM, MPL and the combination at 4 and 8 mg/kg showed marked differences in the two dosage regimens. At the 4 mg/kg dose all 3 groups showed low levels of MØ activation. At the 8 mg/kg dose only the combination treatment induced MØ antitumor activity and decreases in both 5'N and APD specific activities. The NK cell activation potential of TDM and MPL remain to be established. The data with MTP-PE in liposomes varied between the two experiments performed. There appear to be complex relationships between antiviral activity, immunomodulation, and viral pathogenesis that need to be delineated before the mechanisms involved in the antiviral activity can be established.

PUBLICATIONS RESULTING FROM THIS WORK

1. Morahan, P.S., E.R. Leake, D.J. Tenney and M.S. Sit, 1986. Comparative analysis of modulators of nonspecific resistance against microbial infections. In: Immunologic Adjuvants and Modulators of Nonspecific Resistance to Microbial Infections (Ed. J. Majde), Alan R. Liss, NY, In press.

2. Pinto, A.J., P.S. Morahan and M. Brinton, 1986. Comparative study of immunomodulatory activity and antiviral efficacy of various immunomodulators. Abstract, Presented at the ASM Symposium on Host Defenses and Immunomodulation to Intracellular Pathogens, Philadelphia, Pennsylvania.

3. Cosentino, L.M., M.S. Sit and P.S. Morahan, 1986. The permissiveness of B6C3F1 mouse peritoneal macrophages to infection by HSV-1 and HSV-2 and the expression of ICP4 and VP5. Asbstract, Presented at the ASM Symposium on Host Defenses and Immunomodulation to Intracellular Pathogens, Philadelphia, Pennsylvania.

STATEMENT OF PLANS FOR THE NEXT YEAR

During the second year of the current contract we will continue to evaluate compounds for modulation of specific and nonspecific immune responses in normal B6C3F1 mice. We will also continue our studies on the antiviral efficacy of immunomodulator treatment and on the intrinsic MØ resistance in normal and immunomodulator treated mice against a variety of viruses. Based upon the results of our immunomodulator profile and antiviral studies, we will continue to investigate such issues as: dose response, routes of administration, duration of prophylactic antiviral effect, and effectiveness for therapeutic treatment. We also plan to begin some initial experiments with combined agents. Furthermore, to help determine whether the observed antiviral effect of selected immunomodulators is related to alterations in nonspecific immunity, we will examine changes in MØ activation and NK activity in immunomodulator treated mice selectively depleted of MØ and NK cells. We will also examine how this selective depletion alters the antiviral efficacy of the selected agent. Finally, we will complete development of our isolation procedure for Kupffer MØ and their initial characterization. If results progress as we expect, we will begin analysis of immunomodulator effects on Kupffer cells.

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Table 1. Immunomodulators Used in this Study

Compound	General Structure
	Microbially derived
P. acnes (C. parvum)	Killed whole cells
TDM	Trehalose dimycolate
MPL	Detoxified lipid A
MTP-PE	Muramyl tripeptide-phosphatidylethanolamine
	Biologicals
rIFN-A	Human recombinant alpha A/D interferon
rIFN-G	Murine recombinant gamma interferon
rTNF-A	Human recombinant alpha tumor necrosis factor
	Chemicals
MVE-2	Maleic anhydride divinyl ether copolymer
Ampligen	Polyribonucleotide
CL246,738	3,6-bis (2-piperidinoethoxy) acridine trihydrochloride

Table 2. Virus Strains

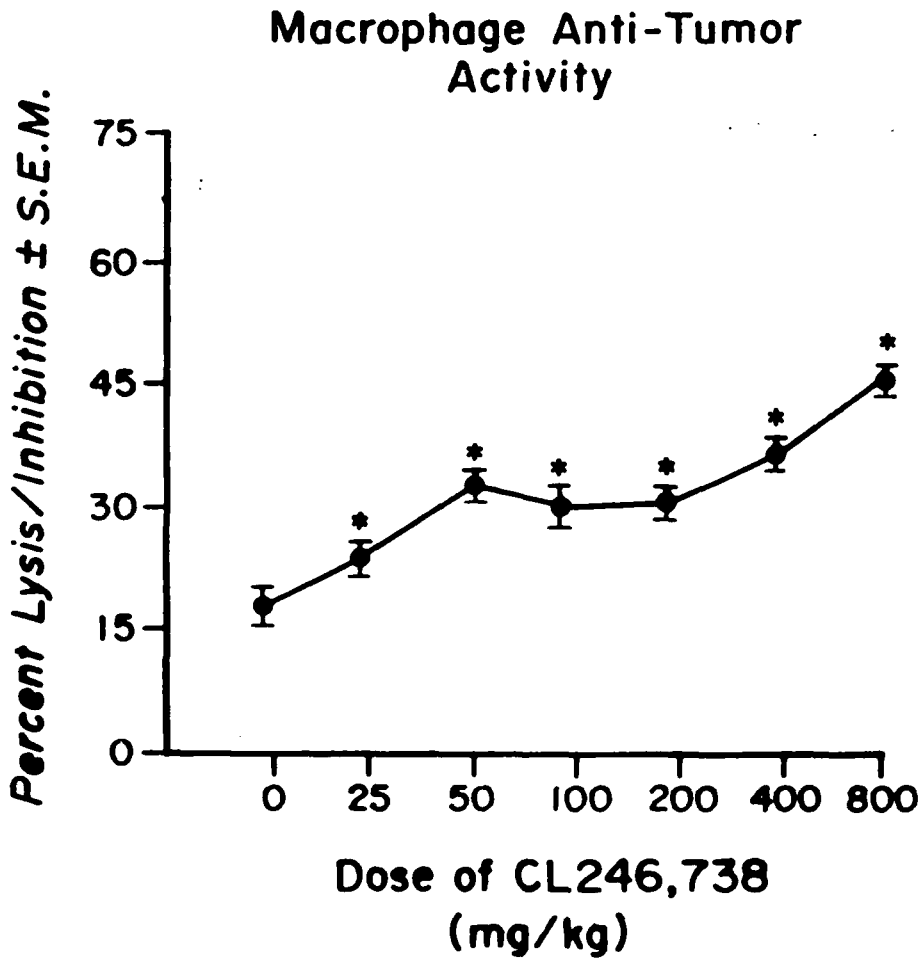
Virus Group	Strain	Source of Pool or Seed	Virus Titers: In vitro PFU/ml	Virus Titers: In vivo i.c.LD_{50} (PFU/LD_{50})	Virus Titers: In vivo i.p.LD (PFU/LD_{50})
Alphaviruses	Sindbis 339	BHK21	$4x10^9$	10^2 ($4x10^6$ PFU/LD_{50})	No symptoms with undiluted
	Sindbis 339	MBP*	2.5×10^9	No symptoms with 10^{-1} dilution	No symptoms with undiluted
	VEE Vaccine strains:	MBP	1.3×10^{10}	Not applicable	$10^{5.0}$ ($1.3x10^4$ PFU/LD_{50})
	Semliki Forest virus L10	MBP	In progress	Not applicable	In progress
Flaviruses	West Nile E101	MBP	1.8×10^9	Not being used *in vivo*	
	Yellow Fever 17D	MBP, one passage	1.6×10^7	3×10^4 ($33PFU/LD_{50}$)	No symptoms with undiluted
	Banzi	MBP	2.0×10^8	Not applicable	$10^{8.4}$ (0.8 PFU/ LD_{50})
Bunyaviruses	Caraparu	MLP	In progress	Not applicable	$10^{2.4}$
	Oriboca	MLP	$4x10^6$ (BHK21)	Not applicable	$10^{1.2}$ ($2.8x10^3$ PFU/LD_{50})
Arenaviruse	Pichinde CoAn3739	Vero	4.5×10^8	Not being used *in vivo*	
Herpesviruses	HSV-2 MS	RKCC	8.0×10^6	Not applicable	ca. $10^{1.0}$ (ca. 1.0×10^5 PFU/LD_{50})

MBP - 10% W/V infant mouse brain pool. MLP - 10% W/V infant mouse liver pool.

Table 3. Effect of CL246,738 on peritoneal macrophage ectoenzyme phenotype

Group	Dose (mg/kg)	Day	# Ectoenzyme Specific Activity (in n moles/min/mg protein) 5'N	APD
Naive	-	-	20.6 ± 2.0	22.6 ± 1.6
C. parvum	35	-7	*1.0 ± 0.4	*2.6 ± 0.2
CL246,738	800	-1	10.5 ± 4.2	12.8 ± 0.6
CL246,738	400	-1	8.7 ± 0.7	*12.2 ± 0.7
CL246,738	200	-1	7.4 ± 0.4	*15.0 ± 0.6
CL246,738	100	-1	18.2 ± 7.5	*12.5 ± 0.5
CL246,738	50	-1	15.0 ± 1.4	*15.9 ± 0.4
CL246,738	25	-1	19.7 ± 4.2	*17.0 ± 1.0
Naive	-	-	21.0 ± 0.4	25.6 ± 0.8
C. parvum	35	-7	*0.4 ± 0.2	*1.7 ± 0.1
CL246,738	800	-4	24.4 ± 6.0	22.4 ± 0.6
CL246,738	800	-3	13.4 ± 0.8	*15.8 ± 0.8
CL246,738	400	-4	18.4 ± 4.3	*17.0 ± 0.8
CL245,738	400	-3	16.3 ± 2.3	*13.2 ± 0.2

C. parvum was inoculated i.p. and CL246,738 was administered per os on the days indicated prior to cell harvest. Each group represents the pool of 5 B6C3F1 mice
± S.E.M., *Decreased activity as compared to naive control, $p < 0.05$.
5'N = 5' Nucleotidase, APD = Alkaline Phosphodiesterase.



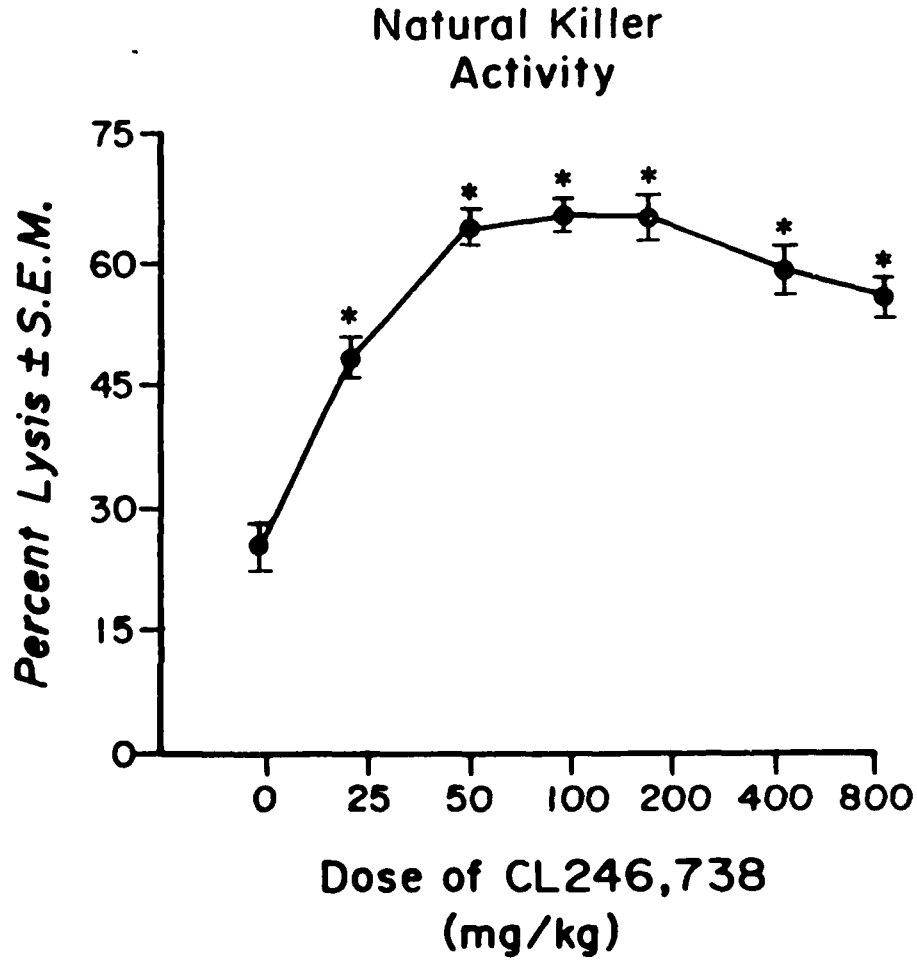


FIGURE 1. DOSE RESPONSE EFFECT OF CL246,738 ON SPLENIC NATURAL KILLER ACTIVITY AND PERITONEAL MACROPHAGE ANTITUMOR ACTIVITY. CL246,738 WAS ADMINISTERED PER OS IN DISTILLED WATER TO B6C3F1 MICE ONE DAY BEFORE CELL HARVEST. *INCREASED ACTIVITY AS COMPARED TO CONTROL MICE WHO RECEIVED DISTILLED WATER ALONE, $P < 0.05$.

Table 4. Kinetics of CL246,738 on splenic natural killer (NK) activity and peritoneal macrophage (MØ) antitumor (AT) activity

Group	Day	NK Activity # (% Lysis) YAC-1 Target	MØ AT Activity # (% Lysis/Inhibition) LL Target
Naive	-	25.8 ± 8.8	18.0 ± 3.0
C. parvum (35mg/kg)	7	ND	*62.3 ± 3.4
CL246,738 (200mg/kg)	-1	*89.8 ± 0.1	18.5 ± 1.3
CL246,738 (200mg/kg)	-4	*77.2 ± 0.6	*27.7 ± 1.5
CL246,738 (200mg/kg)	-7	*65.0 ± 1.5	*30.9 ± 1.6
CL246,738 (200mg/kg)	-10	*61.3 ± 1.1	*27.5 ± 1.3

C. parvum was inoculated i.p. and CL246,738 was administered per os on the days indicated prior to cell harvest. ND = not done, # ± S.E.M., *Increased activity as compared to naive control, $p < 0.05$. Effector to target cell ratios = 200:1 for NK activity and 10:1 for MØ AT Activity. Each group represents the pool of 5 B6C3F1 mice.

Table 5. Dose response effect of ampligen on splenic natural killer (NK) activity and peritoneal macrophage (MØ) antitumor activity (AT) and ectoenzyme specific activity (SA).

Group	NK Activity # (% Lysis) YAC-1 Target	MØ AT Activity # (% Lysis/Inhibition) LL Target	Ectoenzyme SA # (n moles/min/mg protein) 5'N	APD
Naive	25.9 ± 0.6	31.6 ± 1.4	20.9 ± 5.8	20.4 ± 0.9
C. parvum (35mg/kg)	ND	*99.6 ± 0.4	*0.7 ± 0.3	*2.8 ± 0.9
Ampligen (8mg/kg)	*60.0 ± 1.0	*62.2 ± 1.7	*5.1 ± 1.2	*8.2 ± 0.8
Ampligen (4mg/kg)	*59.8 ± 1.9	*49.6 ± 1.7	*4.3 ± 0.3	*8.2 ± 0.8
Ampligen (2mg/kg)	*56.2 ± 0.7	*46.6 ± 3.2	*4.6 ± 0.6	*9.3 ± 1.0
Ampligen (1mg/kg)	*58.0 ± 1.1	38.1 ± 2.0	*3.4 ± 0.6	*9.8 ± 2.4

B6C3F1 mice were injected i.p. with C. parvum 7 days before sacrifice, or 1 day before sacrifice with the indicated dose of ampligen. ND = not done, 5'N = 5' nucleotidase, APD = alkaline phosphodiesterase. *Significantly different as compared to naive control, $p < 0.05$. # ± S.E.M. Effector to target cell ratios = 200:1 for NK activity and 20:1 for MØ AT activity. Each group represents the pool of 5 B6C3F1 mice.

Table 6. Dose response effect of gamma interferon (rIFN-G) on splenic natural killer (NK) activity and peritoneal macrophage (MØ) antitumor (AT) activity and ectoenzyme specific activity (SA).

Group	NK Activity (% Lysis) YAC-1 Target	MØ AT Activity (% Lysis/Inhibition) LL Target	Ectoenzyme SA (n moles/min/mg protein) *5'N	#APD
Naive	27.3	44.5	32.5	35.2
Vehicle (0.2% BSA)	37.2	49.8	67.1	21.5
C. parvum (35 mg/mg)	ND	100.0	0.5	4.0
rIFN-G (22,000 U)	67.0	100.0	5.5	5.2
rIFN-G (6,000 U)	64.5	100.0	8.4	9.9
rIFN-G (300 U)	55.9	100.0	2.8	12.1
rIFN-G (30 U)	44.0	100.0	3.7	15.2

B6C3F1 mice were injected i.p. with C. parvum 7 days before sacrifice, or 1 day before sacrifice with the indicated dose of rIFN-G. ND = not done, *5'nucleotidase, #alkaline phosphodiesterase. Effector to target cell ratios = 200:1 for NK activity and 20:1 for MØ AT activity. Each group represents the pool of 3 B6C3F1 mice.

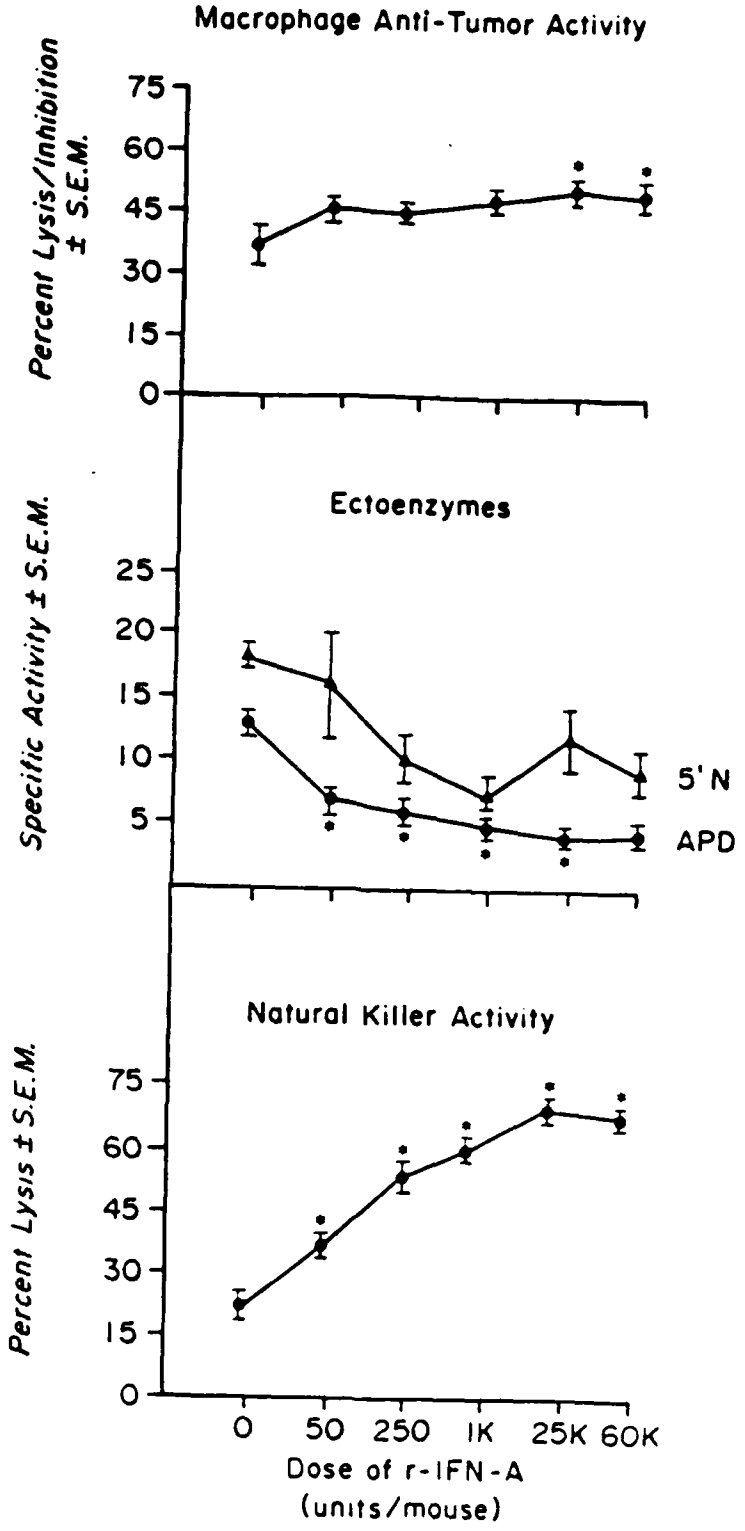


Figure 2. Dose response effect of human recombinant alpha A/D interferon (rIFN-A) on splenic natural killer activity and peritoneal macrophage anti-timor activity and ectoenzyme specific activity. B6C3F1 mice were injected i.p. one day before sacrifice with the indicated dose of rIFN-A. *Statistically different from control mice, $p < 0.05$.

Table 7. Effect of trehalose dimycolate (TDM) and monophosphoryl lipid A (MPL) on peritoneal macrophage antitumor activity and ectoenzyme phenotype

Group	Dose (mg/kg)	MØ Antitumor Activity (Percent Lysis/Inhibition) LL Target	Ectoenzyme Specific Activity (in n moles/min/mg protein)	
			5'N	APD
Naive	-	25.2	18.0	21.4
Vehicle	-	28.2	13.3	18.4
C. parvum	35	100.0	0.2	1.8
MPL	4	48.6	21.7	8.7
TDM	4	39.6	14.1	9.8
MPL + TDM	4 + 4	43.5	4.1	4.9
Naive	-	0.1	27.3	31.3
Vehicle	-	7.1	13.7	27.8
C. parvum	35	100.0	0.4	2.1
MPL	8	13.3	4.0	5.0
TDM	8	3.4	26.2	21.3
MPL + TDM	8 + 8	52.7	6.4	9.0

B6C3F1 mice were inoculated with the indicated agents. Peritoneal cells were harvested 7 days later from pools of 5 mice. 5'N = 5'nucleotidase, APD = alkaline phosphodiesterase.

Table 8. Effect of MTP-PE on Peritoneal Macrophage Ectoenzymes

Groups	Cells/Mouse x 10^6	Specific Activity (n moles/min/mg protein) 5'N	APD Exp 1	APD Exp 2
Naive	3.6	36.4	17.1	27.8
Pyrogen Free NaCl	5.3	27.3	14.8	21.4
Sham Lipsomes	15.3	1.0	18.6	46.7
Free MTP-PE	3.6	18.3	7.6	12.6
Sham Liposomes & Free MTP-PE	12.5	1.2	20.0	38.9
Liposomes containing MTP-PE	28.5	1.2	2.3	39.0
C. parvum	30.0	0.2	3.7	4.8

Mice were injected i.p. with C. parvum (56 mg/kg) 5 days before sacrifice, or 2 days before with MTP-PE (100ug/mouse). The ectoenzyme SA were determined for pools of peritoneal macrophages. 5'N = 5'nucleotidase, APD = alkaline phosphodiesterase.

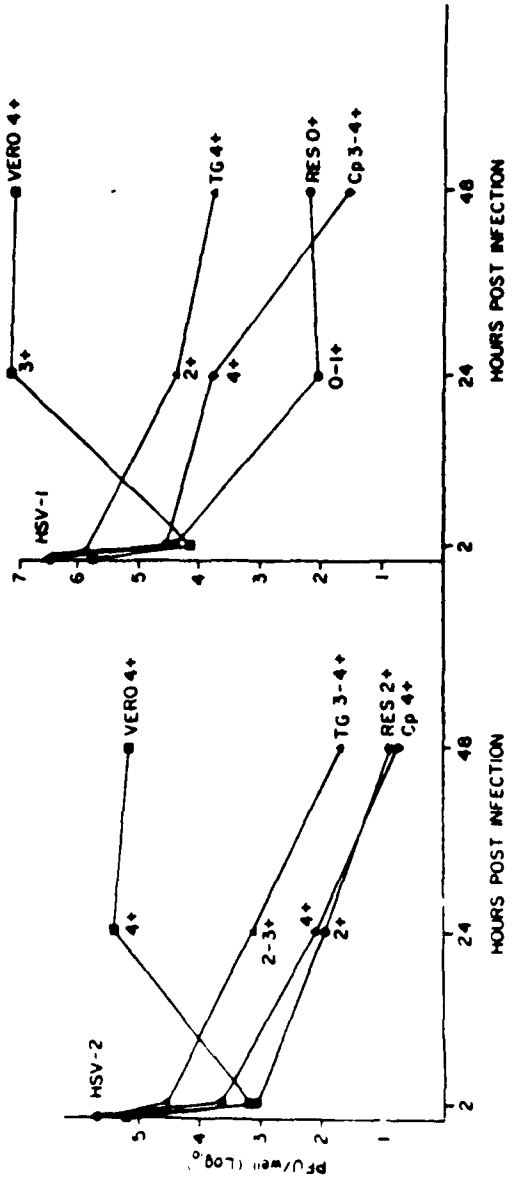


FIGURE 5. INFECTIOUS VIRUS YIELDS FROM B6C3F1 MOUSE RESIDENT (RES) MΦ, C. PARVUM (Cp) MΦ, THIOGLYCOLLATE MΦ (TG), AND VERO CELLS FOLLOWING INFECTION WITH HSV-1 STRAIN K OS. RESULTS ARE GIVEN AS Log_{10} PFU/WELL YIELDS AND CPE (+1 TO +4).

Table 9. Intrinsic Interaction of Viruses with Res Peritoneal MØ

Virus	m.o.i.		Hours After Infection of B6C3F1 MØ 24	48	72
HSV-1 KOS	5.0	PFU/cell*	0.0006	0.0001	ND
		CPE**	0-1+	0+	ND
HSV-2 MS	2.0	PFU/cell	0.0001	0.0001	ND
		CPE	1-2+	1-2+	ND
Pichinde	1.0	PFU/cell	0.003	ND	0.005
		CPE	0	0	0-1+
YFV	0.3	PFU/cell	0.0001	0.0001	0.0001
		CPE	4+	4+	4+
Sindbis	3.0	PFU/cell	0.16	0.003	0.0003
		CPE	0-1+	0-1+	0-1+
Oriboca	0.003	PFU/cell	0.019	0.16	ND
		CPE	0	0	0

Res MØ were cultured for 24 hr and infected with viruses at the m.o.i. indicated.

* Infectious PFU produced per MØ, calculated from the yield divided by the actual or estimated number of MØ present at the time of infection. HSV and Pichinde yields were total (cells + supernatant) while the other virus yields are for released virus. ND = Not done.

**CPE was evaluated microscopically, with 0 -1 + = 0-25% CPE, 1-2 + = 25-50% CPE, and 4 + = 100% CPE.

Table 10. Kinetics of Production of HSV-1 Antigens after Infection of Various Cells

Cells	Antigen	Percent Positive Cells Hours after Infection				
		1	3	6	12	24
Vero	ICP4	0 (1)	44 (3)	73 (3)	ND	97 (3)
	VP5	0 (1)	15 (3)	55 (3)	ND	96 (4)
Res	ICP4	0 (3)	8 (4)	12 (4)	17 (2)	ND
	VP5	3 (3)	15 (5)	23 (4)	44 (2)	38 (2)
TG	ICP4	0 (3)	6 (4)	16 (3)	25 (2)	ND
	VP5	1 (3)	6 (5)	19 (4)	62 (2)	ND
Cp	ICP4	0 (1)	7 (2)	10 (2)	ND	ND
	VP5	2 (3)	9 (4)	12 (2)	ND	ND

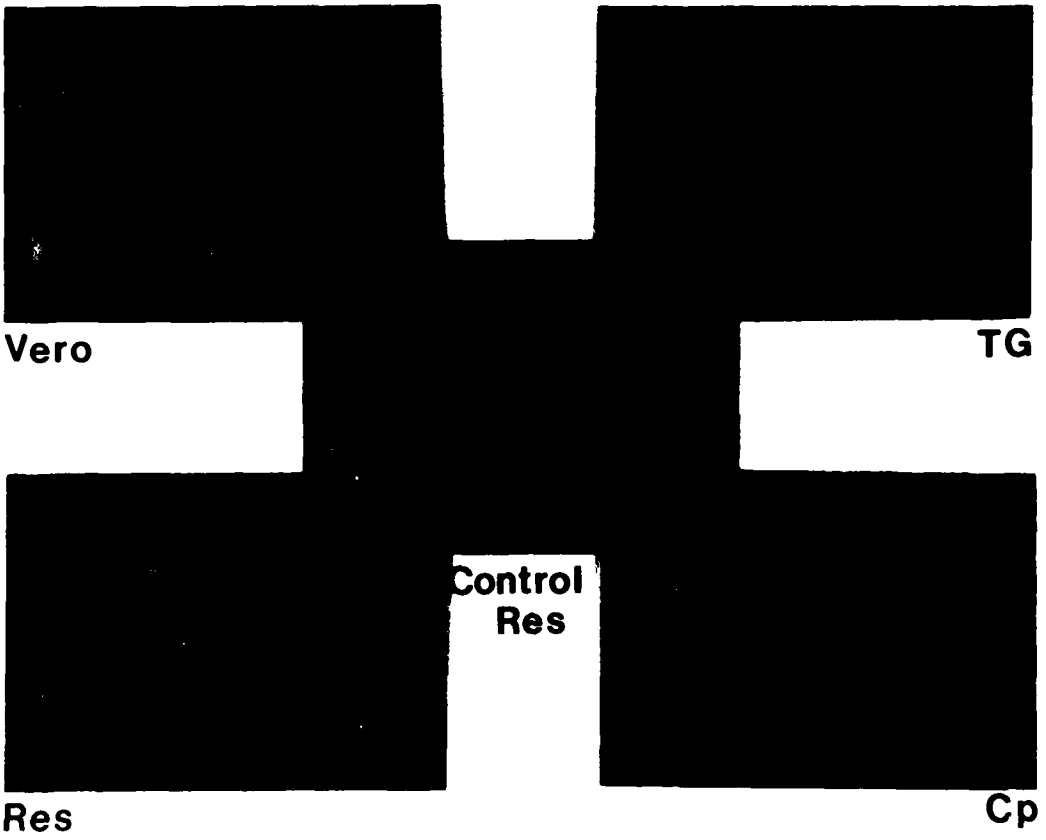


Figure 4. Immunofluorescence with anti-ICP4 6 hours post infection of Vero cells and B6C3F1 mouse resident (Res), C. parvum (Cp) and thioglycollate (TG) peritoneal macrophages with HSV-1 strain Kos. Control Res = uninfected Res peritoneal macrophages.

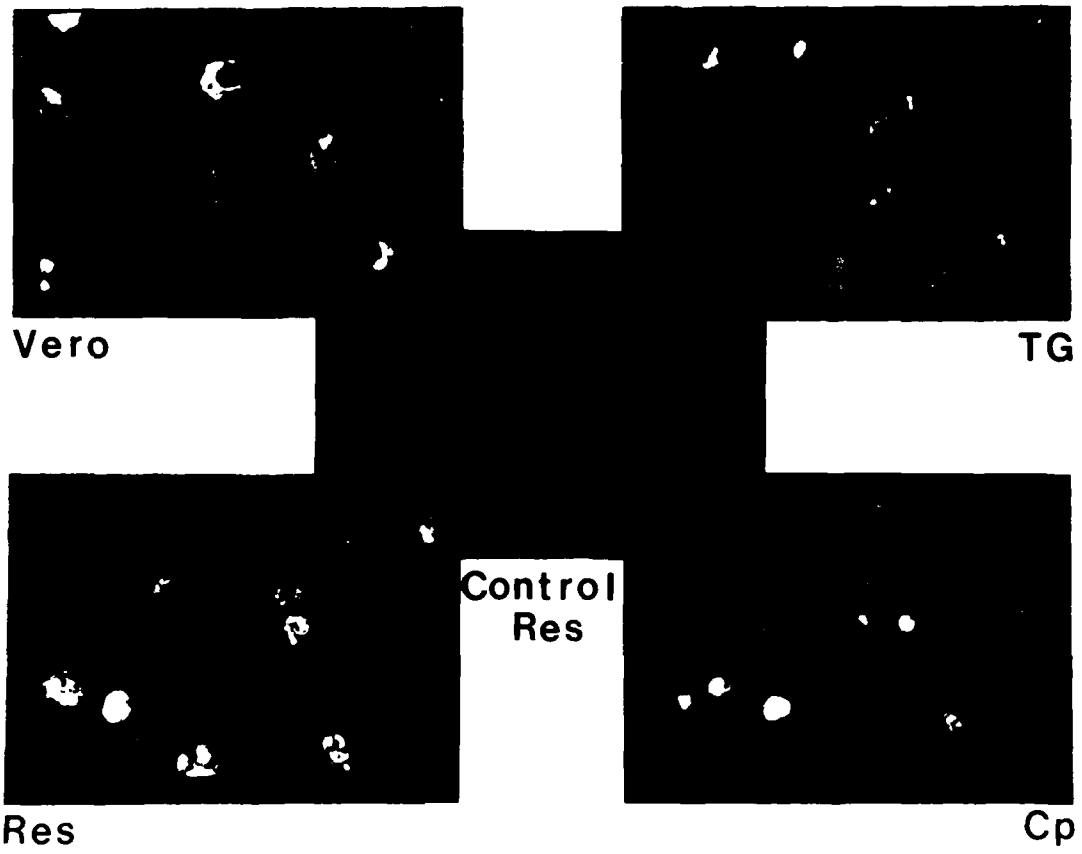


Figure 5. Immunofluorescence with anti-VP5 6 hours post infection of Vero cells and B6C3F1 mouse resident (Res), C. parvum (Cp) and thioglycollate (TG) peritoneal macrophages with HSV-1 strain Kos. Control Res = uninfected Res peritoneal macrophages.

Table 11. Lack of Antiviral Activity of Immunomodulators Against Intracerebral Infection with Yellow Fever Virus (YFV) and Sindbis Virus (SV)

Regimen				Mortality		Survival
Drug	Dose	Schedule (days)	Route	Dead/ Total	(%)	MST (days)
A. Yellow Fever Virus Challenge						
PBS/0.2% BSA	(0.2 ml)	D-0toD+7	i.p.	15/15	(100%)	10.1
IFN-G	43,400 U	D-0toD+7	i.p.	9/10	(90%)	10.7
IFN-B	360 U	D-0toD+7	i.p.	10/10	(100%)	9.2
IFN-A	61,400 U	D-0toD+7	i.p.	10/10	(100%)	10.0
C. parvum	35 mg/kg	D-7	i.p.	10/10	(100%)	9.7
MVE-2	50 mg/kg	D-7	i.p.	10/10	(100%)	8.5
MVE-2	50 mg/kg	D-1	i.p.	9/10	(90%)	9.4
CL246,738	400 mg/kg	D-1	i.p.	9/10	(90%)	9.6
CL246,738	200 mg/kg	D-1	i.p.	10/10	(100%)	9.3
2 % Squalene	(0.2 ml)	D-7	i.p.	9/10	(90%)	9.8
MPL	8 mg/kg	D-7	i.p.	10/10	(100%)	8.5
TDM	8 mg/kg	D-7	i.p.	10/10	(100%)	9.1
MPL+TDM	8 mg/kg	D-7	i.p.	9/9	(100%)	9.8
B. Sindbis Virus Challenge						
PBS/0.2% BSA	(0.2ml)	D-7	i.p.	12/14	(86%)	8.1
C. parvum	35 mg/kg	D-7	i.p.	5/5	(100%)	8.6
MVE-2	50 mg/kg	D-7	i.p.	5/5	(100%)	7.4

B6C3F1 mice were treated with the indicated doses (mg/kg or units for IFNs) and schedule (days) in relation to challenge with about 120 PFU (20 LD_{50} doses) of YFV or 5 x 10^5 PFU of SV. Cumulative mortality and mean survival time (MST) were calculated and compared with the appropriate vehicle/control groups, by Chi square (mortality) and Mann-Whitney U test (MST).

Table 12. Effects of Immunomodulators on Infection with HSV-2 MS Virus

Drug Treatment					Mortality		Survival
Drug	Dose (mg/kg)	Schedule	Route	Vehicle	Dead/ Total	(%)	MST
Placebo/IFN Placebo	-	0/-1 to +6 (2x)	i.p.	GLB/PBS/0.2%BSA	30/33	(91%)	10.3
C. parvum	35	-7	i.p.	NaCl	0/10	(0%)*	22.0*
MVE-2	50	-1	i.p.	PBS	0/10	(0%)*	22.0*
TDM+MPL	0.02+0.008	-7	i.p.	H_2O	4/10	(40%)*	17.0*
WS+MPL+TDM	0.008+0.008+0.008	-7	i.p.	H_2O	4/10	(40%)*	17.2*
TDM	0.04	-7	i.p.	H_2O	4/10	(40%)*	16.7*
STM	0.008	-7	i.p.	H_2O	4/10	(40%)*	17.5*
PA-PE	0.1	-7	i.p.	H_2O	5/10	(50%)	14.9
S-209	0.125	-7	i.p.	H_2O	7/10	(70%)	12.4
rIFN-A	66,000 IU	-1 to +6 (2x)	i.p.	PBS/0.2%BSA	0/10	(0%)*	22.0*
rIFN-A	66,000 IU	-1 to +6 (1x)	i.p.	PBS/0.2%BSA	0/10	(0%)*	22.0*
rIFN-A	66,000 IU	-1 to +2 (2x)	i.p.	PNS/0.2%BSA	0/10	(0%)*	22.0*
Placebo	-	0	i.p.	GLB	16/30	(53%)	15.6
C. parvum	35	-7	i.p.	NaCl	0/10	(0%)*	24.0*
MVE-2	50	-1	i.p.	PBS	0/10	(0%)*	24.0*
CL246,738	50	-1	p.os	H_2O	1/10	(10%)*	22.7*
CL246,738	25	-1	p.os	H_2O	1/10	(10%)*	22.4*
CL246,738	12.5	-1	p.os	H_2O	6/10	(60%)	14.6
CL246,738	200	0	p.os	H_2O	1/9	(11%)*	21.2*
CL246,738	200	-1	p.os	H_2O	2/10	(20%)	19.5
CL246,738	200	-2	p.os	H_2O	2/10	(20%)	19.3
Ampligen	4	-1	i.p.	NaCl	10/20	(50%)	17.7
Ampligen	8	-1	i.p.	NaCl	3/10	(30%)	18.4
Ampligen	4	-1 to +6 (1x)	i.p.	NaCl	0/10	(0%)*	22.0*

B6C3F1 female mice, aged 6-10 wks old, were treated as indicated and infected on day 0 i.p. with 1.2 to 6.6 x 10^5 PFU (1-3 LD_{50} doses) of HSV-2 MS virus.

* = Statistically significant, $p < 0.05$.

Table 13. Effect of Immunomodulators on Banzi Flavivirus Infection

Drug Treatment					Mortality		Survival
Drug	Dose (mg/kg)	Schedule (days)	Route	Vehicle	Dead/ Total	(%)	MST
Placebo	-	0	i.p.	NaCl	29/30	(97%)	9.8
MVE-2	50	-1	i.p.	PBS	0/16	(0%)*	19.0*
C. parvum	35	-7	i.p.	NaCl	0/10	(0%)*	19.0*
CL246,738	50	-1	p.os	H_2O	0/10	(0%)*	19.0*
CL246,738	25	-1	p.os	H_2O	3/10	(30%)*	17.1*
CL246,738	12.5	-1	p.os	H_2O	1/10	(10%)*	19.0*
CL246,738	200	-4	p.os	H_2O	10/10	(100%)	11.2*
CL246,738	200	-1	p.os	H_2O	0/20	(0%)*	19.0*
CL246,738	200	0	p.os	H_2O	7/10	(70%)	15.4*
CL246,738	200	+1	p.os	H_2O	8/10	(80%)	13.2*
CL246,738	200	+3	p.os	H_2O	10/10	(100%)	8.0
CL246,738	200	+1	i.p.	H_2O	10/10	(100%)	7.3
Ampligen	4	0 to +6	i.p.	NaCl	1/5	(20%)*	17.8*
Ampligen	4	0	i.p.	NaCl	0/20	(0%)*	19.0*
Ampligen	4	+1	i.p.	NaCl	9/9	(100%)	9.7
IFN Placebo	-	0 to +6	i.p.	PBS/0.2% BSA	20/22	(91%)	9.5
rIFN-G	50,000 IU	0 to +6	i.p.	PBS/0.2% BSA	7/10	(70%)	13.8
rIFN-A	20,000 IU	0 to +6	i.p.	PBS/0.2% BSA	0/10	(0%)*	19.0*
rIFN-A	24,400 IU	0	i.p.	PBS/0.2% BSA	4/10	(40%)*	17.5*
rTNF-A	20,000 IU	0 to +6	i.p.	PBS/0.2% BSA	10/10	(100%)	8.2

B6C3F1 female mice, aged 6-10 wks old, were treated as indicated, and infected on day 0 i.p. with ca. 4 PFU of (20 LD_{50} doses) Banzi virus.

* = Statistically significant $p < 0.05$.

Table 14. Effect of Immunomodulators on Infection with VEETC83 Vaccine Strain Alphavirus

Drug Treatment					Mortality		Survival
Drug	Dose (mg/kg)	Schedule (days)	Route	Vehicle	Dead/ Total	(0%)	MST (days)
Placebo	-	0	i.p.	NaCl	19/20	(95%)	13.0
MVE-2	50	-1	i.p.	PBS	5/16	(32%)	21.2*
C. parvum	35	-1	i.p.	NaCl	9/11	(82%)	14.2
CL246,738	100	-1	p.os	H_2O	0/10	(0%)*	26.0*
Ampligen	4	-1 to +6	i.p.	NaCl	1/10	(10%)*	24.6*
Ampligen	4	-1 to +2	i.p.	NaCl	0/10	(0%)*	22.0*
Ampligen	4	-1	i.p.	NaCl	7/10	(70%)	15.2
Ampligen	4	0 to +6	i.p.	NaCl	5/10	(50%)	21.1*
IFN Placebo	-	-1 to +6	i.p.	PBS/0.2%BSA	19/22	(86%)	13.8
rIFN-G	50,000 IU	-1 to +6	i.p.	PBS/0.2%BSA	2/10	(20%)*	20.7*
rIFN-G	50,000 IU	0 to +6	i.p.	PBS/0.2%BSA	8/10	(80%)	17.5*
rIFN-A	24,400 IU	0	i.p.	PBS/0.2%BSA	9/10	(90%)	12.6
rIFN-A	24,000 IU	0 to +6	i.p.	PBS/0.2%BSA	1/10	(10%)*	21.2*
rTNF-G	10,000 IU	o to +6	i.p.	PBS/0.2%BSA	9/10	(90%)	12.8

B6C3F1 female mice, aged 6-10 wks old, were treated as indicated, and infected on day 0 i.p. with 1.6×10^5 PFU (7 LD_{50} doses) of VEE TC83 strain.

* = Statistically significant $p < 0.05$.

Table 15. Effect of Various Immunomodulators on Caraparu Bunyavirus

Drug Treatment					Mortality		Survival
Drug	Dose (mg/kg)	Schedule (days)	Route	Vehicle	Dead/ Total	(%)	MST (days)
Placebo	-	0	i.p.	GLB	10/10	(100%)	4.2
MVE-2	50	-1	i.p.	PBS	4/10	(40%)	11.0*
CL246,738	200	-1	p.os	H_2O	9/10	(90%)	7.8*
CL246,738	100	-1	p.os	H_2O	8/10	(80%)	7.7*
CL246,738	50	-1	p.os	H_2O	9/10	(90%)	5.5
Ampligen	4	-1	i.p.	NaCl	9/10	(90%)	5.4
Ampligen	2	-1	i.p.	NaCl	8/10	(80%)	6.3

B6C3F1 female mice, aged 6-10 weeks were treated and infected on day 0 i.p. with 20 LD_{50} doses of Caraparu virus.

* = Statistically significant $p < 0.05$.

END

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